PREPARATION OF DERMATOPHYTES FOR AMINO ACID RESPIRATORY STUDIES*

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Unlike other filamentous fungi, such as various species of penicillium, aspergillus and neurospora, comparatively little is known concerning the metabolic systems of the superficial dermatophytes. Physiological studies on these organisms have been partly curtailed, as a result of their high endogenous activity. Attempts to reduce this activity by starvation often results in loss of some enzymic functions and not endogenous activity alone. As a result, limited studies on amino acid metabolism have been reported (1, 2).

In general, dermatophytes alter the pH of their environment by an alkaline reaction (3, 4, 5, 6, 7), and this change is coincident with ammonia production. Subsequently, Burack and Knight (2) have shown that a wide range of amino acids may be deaminated by these organisms, and have stated that the microenvironment of a dermatophyte infected area would have a tendency to become quite alkaline. Further, they suggested that this alteration in pH in the infected area could contribute to the infectious process. Although an alkaline environment could contribute to the initiation of concurrent secondary bacterial infections, amino acid deamination by dermatophytes has received but cursory investigation. For this reason, further studies on this topic appear warranted.

This investigation revealed a starvation method which reduced endogenous activity of certain strains of dermatophytes permitting quantitative respiratory studies. Certain observations from these metabolic studies are reported as the subject of this paper.

MATERIALS AND METHODS

Organisms: Three strains of *Trichophyton mentagrophytes*, #666, #675 and #687 were obtained through the courtesy of Dr. C. W. Emmons, Bethesda, Md. *T. mentagrophytes*, #299.2 was obtained from Dr. M. Silva, Columbia University. The additional organisms tested were obtained from Dr. F. Blank, Departments of Dermatology and Microbiology, Temple University, Philadelphia, Pa. All organisms have been maintained during the past five years in this Department on Sabouraud's glucose agar.

Submerged Growth Cultivation: Growth from the surface of a Sabouraud's glucose agar slant was fragmented and used as a disperse inoculum for 50 ml of Sabouraud's glucose medium containing 0.2 ml of Triton X-100 (1%) in a 500 ml Erlenmeyer flask. Incubation was carried out for 3 days at room temperature on a Burrell wrist-action shaker at 225 cycles per minute with a 4 cm stroke.

Ten ml of this growth served as inoculum for each 500 ml flask containing 50 ml of the aforementioned liquid medium. After four days incubation at room temperature on the shaker, growth in the form of small pellets (average 2 mm diameter) was harvested by centrifugation at 1,200 × g for 30 minutes. The sedimented growth was placed in and washed three separate times with sterile physiological saline, and then resuspended in sterile 0.066 M phosphate buffer, pH 7.0.

This procedure was followed in the preparation of growth of *Microsporum quinckeanum*, #8. Slightly longer incubation periods were required for the growth of *M. quinckeanum*, #13 and for other species tested.

Starvation of Cell Preparations: The nature and duration of the starvation period employed varied according to the degree of starvation desired; *e. g.*—fragmentation of pellets and mycelia, the organism used, and whether enhancement in deaminase activity was desired through the addition of L-alanine.

The following example is given to illustrate the procedure used in reducing endogenous activity of *M. quinckeanum*, #8 and concurrent fragmentation of pellets obtained from submerged growth cultivation. A similar approach was used in reduction of endogenous activity for other organisms used. Using *M. quinckeanum*, #8, it is to be noted that the more concentrated the pellet suspension used during starvation, the more readily fragmentation of pellet formation and mycelial elements occurred.

Washed pellets from submerged growth cultivation were obtained from four culture flasks and resuspended in 150 ml of 0.066 M phosphate buffer, pH 7.0. Fifty ml of this suspension were transferred aseptically to each of three 500 ml Erlenmeyer flasks. To each flask was added 0.2 ml of Triton X-100 (1%), 250 I.U. of crystalline potassium penincillin G, and 2,500 μg of streptomycin HCl.

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Antibiotics were used according to results of Raubitschek (8). Prior to use of organisms, sterility tests on each preparation were made by microscopic as well as cultivation technics to determine the presence of possible bacterial contamination.

Reduction in endogenous activity was obtained after starvation had been carried out on the shaker at room temperature for 3 days. The cells were sedimented by centrifugation and the buffer, Triton X-100 and antibiotics were changed. Starvation of cells on the shaker was continued for 2 additional days. Endogenous activity was reduced also by starving cells for 24 hours in buffer, adding 10 μM/ml L-alanine and continuing starvation for 36 hours. Cells were placed then in fresh buffer containing 5 μM/ml L-alanine for 16 hours. In both procedures, after the final starvation step, the cells were washed 3 times with sterile physiological saline and resuspended in 30 ml of 0.066 M phosphate buffer, pH 7.0. These were the materials used for respiratory studies. Manometric measurements were carried out in a Warburg apparatus at 30 C. to determine O_2 consumption. Each flask contained 1.5 ml of cell suspension (5 mg dry wt of cells), 0.066 M phosphate buffer (pH 7.0), and substrate, ammonia, cofactors or inhibitors when required, to a total volume of 3 ml.

Enzyme Preparations: Washed, drained, recently harvested and chilled pellets were ground with a mixture of powered dry ice and fine glass beads (Glass Homogenizing Beads, VirTis Company) in a mortar according to methods previously described (9, 10).

A refrigerated Nossal cell fractionator was used also to disrupt cells. Ten gm of beads were added to 5 gm of wet, packed cells in presence of 2 ml of phosphate buffer containing 0.1 M mercaptoethanol. Excellent breakage of cells was obtained after four shaking periods of 30 seconds each. Before each 30 second interval of operation with concurrent use of liquid CO_2 jet operation on the capsule, a 10 minute period of cooling at −20 C. was employed.

Analytical Methods: Activity of intact organisms and enzyme preparations were determined by Thunberg and Warburg technics (11). Determinations for ammonia, amino and keto acids were made on supernatant aliquots obtained from Warburg flasks. Production of ammonia was determined by the microdiffusion method of Conway (12). Determination of amino acids was made according to the method described by Yemm and Cocking (13). Measurement of keto acids content in supernatants was made by the micro method of Friedmann (14). Chromatography of 2,4-dinitrophenylhydrazone derivatives of keto acids was carried out by the method of El Hawari and Thompson (15).

RESULTS

Ammonia Production

(a) *Starvation of Organisms:*

Endogenous activity was reduced and metabolic studies were possible by use of starvation techniques developed during this investigation. As shown in Figure 1, a marked separation be-

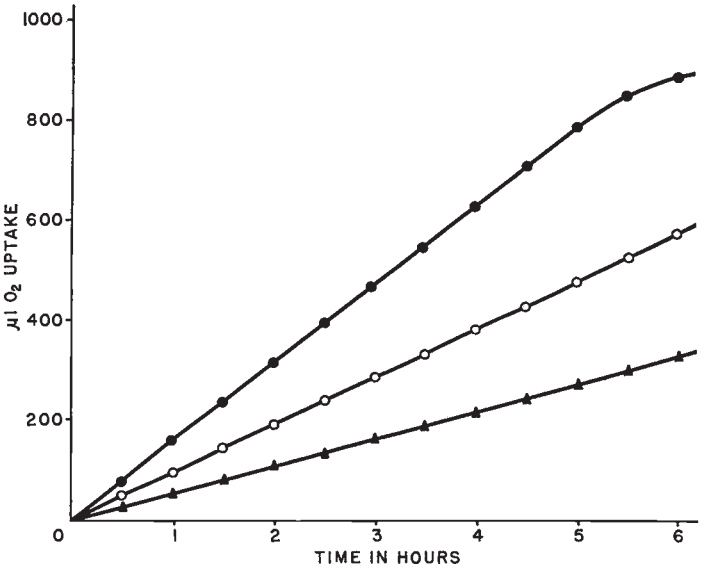

FIG. 1. The effect on the time course of O_2 uptake by the starvation of *M. quinckeanum*, #8, in the presence or absence of 10 μM/ml (36 hours) and 5 μM/ml (16 hours) L-alanine. 1 μM/ml L-alanine, cells starved with alanine (●——●). 1 μM/ml L-alanine, cells starved without alanine (○——○). Endogenous, cells starved in absence or presence of L-alanine (▲——▲). 1.5 ml pellets (dry wt 5 mg/1.5 ml) per flask.

tween endogenous and reaction specimens could be obtained with *M. quinckeanum*, #8, when inclusion of L-alanine was made during a three day starvation period. Pellets starved for three days without added L-alanine did not produce as marked a separation. As graphically reported, oxygen uptake values between endogenous and substrate specimens may be differentiated after 30 minutes reaction time.

Micromoles ammonia produced during respiratory studies with *M. quinckeanum*, #8, starved in the presence of added L-alanine, were 4.6 times greater than that produced by endogenous mycelia. A 20-fold increase in ammonia production was observed in substrate flasks during the reaction times of 30 minutes and 5.5 hours.

During the investigation, several observations were obtained. The inclusion of Triton X-100, penicillin and streptomycin in the concentrations used showed no inhibition of the enzymic activities tested. It was found that the use of cells which had not been starved provided no separation in tests for oxygen uptake between reaction mixtures and those of endogenous flasks. Furthermore, with the organism used, the optimum pH for measurement of respiratory activity was 7.0. Tests with the D-isomer of various amino acids showed no utilization or deamination of these substrates. Tests of supernatants in which pellets had been starved in the presence of 10 μM/ml L alanine for 36 hours produced 11 μM NH_3/ml. After additional starvation in fresh suspending fluid with 5 μM/ml L-alanine, 6.7 μM NH_3/ml were produced. Cells starved for comparable periods in absence of alanine produced 1.4 μM NH_3/ml.

(b) *Amino Acid Oxidase Activity:*

On the basis of rate per mg of dry mycelium, results reported in Table I show that all amino acids tested were deaminated by the dermatophytes used, but in varying degrees. Strain as well as species differences in activity may be noted. In addition, oxygen uptake and ammonia production values differed according to substrates tested. For example, phenylalanine is oxidized readily while NH_3 production is low. Moreover, ammonia production from alanine and aspartic acids is relatively high for all dermatophytes tested. The strain of *E. floccosum* used had a low metabolic activity, and ammonia determinations were not attempted. From the results reported in Table I, the organisms *M. quinckeanum*, #8, and *T. mentagrophytes* #666 and #687 were selected for further study in this investigation, as they actively took up oxygen and deaminated a variety of amino acids.

Fragmented Cellular Preparations

It was noted during this investigation that if starvation times of pellets were extended from 4 days to 5 days a morphological change occurred in

TABLE I

μl O_2 uptake and NH_3 production by dermatophytes in presence of various amino acids

Substrate	Organism													
	M. quinckeanum				*Trichophyton mentagrophytes*								*Epidermophyton floccosum*[1]	
	#8		#13		#675		#687		#666		#299.2			
	μl O_2	μM NH_3/ml	μl O_2	μM NH_3/ml	μl O_2	μM NH_3/ml	μl O_2	μM NH_3/ml	μl O_2	μM NH_3/ml	μl O_2	μM NH_3/ml	μl O_2	μM NH_3/ml
DL-alanine	740	1.28	202	.97	241	.97	420	1.60	311	.97	189	.79	60	—
DL-phenylalanine	712	.61	185	.65	155	.36	334	.76	255	.41	136	.37	48	—
L-leucine	679	.65	151	.63	108	.45	305	.73	214	.39	129	.28	41	—
DL-isoleucine	645	.54	166	.58	130	.36	341	.63	284	.41	110	.30	44	—
DL-valine	571	.53	168	.61	154	.43	340	.85	245	.49	133	.37	43	—
L-glutamic acid	525	.88	169	.63	184	.56	254	.91	251	.67	164	.61	50	—
DL-aspartic acid	495	1.04	181	.92	179	1.15	232	1.19	226	.90	160	.99	52	—
Endogenous	364	.36	145	.54	78	.27	210	.45	193	.27	92	.21	37	.12

Reaction time 6 hours. 1.5 ml cells per flask (5mg dry wt). Not corrected for endogenous. Except for *M. quinckeanum*, #8, all cellular preparations are pellet form of growth. Concentration of substrate (L-isomer) 5μM/ml.

[1] Ammonia determinations not made, as activity was not noted above endogenous.

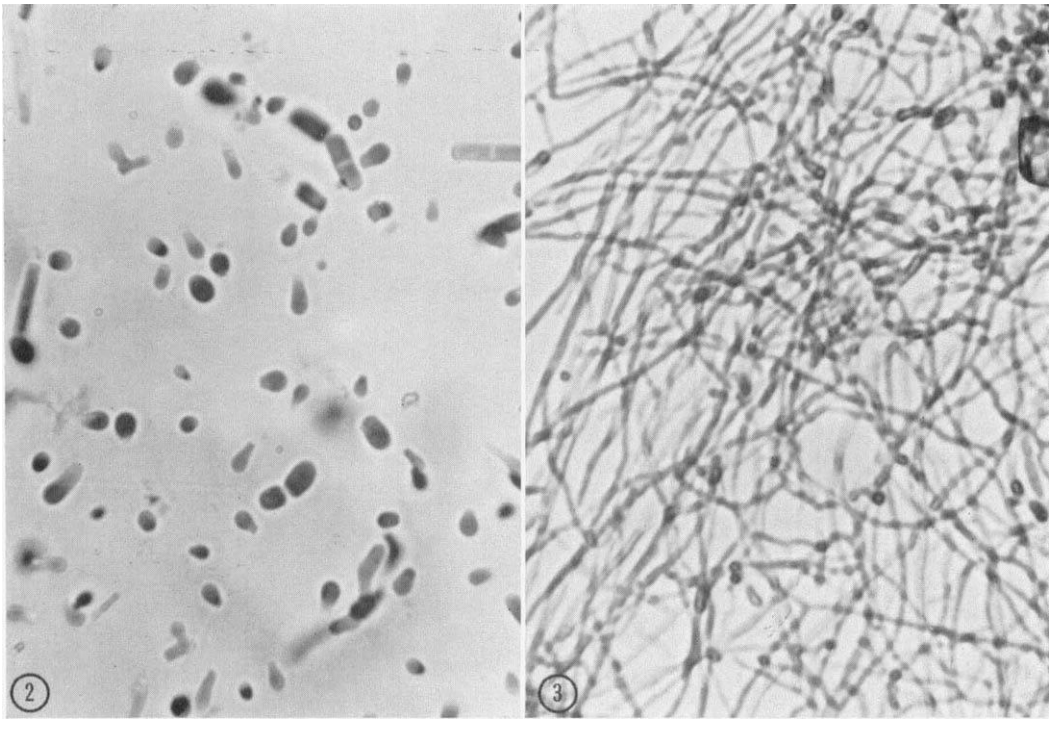

FIG. 2. Morphological appearance of *Microsporum quinckeanum*, #8, after 5 days starvation (acid fuchsin). × 341.

FIG. 3. Morphological appearance of regenerated mycelia from fragmented pellets of *Microsporum quinckeanum*, #8, after 2 days cultivation in Sabouraud's glucose medium (acid fuchsin). × 248.

TABLE II

The effect of fragmentation of pellets on the respiration of Microsporum quinckeanum, #8

Cellular Preparation	$\mu l\ O_2$	$\mu M\ NH_3/ml$
Fragmented mycelia (5 μM/ml L-alanine)	740	1.28
Non-fragmented mycelia (5 μM/ml L-alanine	422	.69
Endogenous fragmented mycelia	364	.36
Endogenous non-fragmented mycelia	231	.21

Reaction time 6 hours. Values based on 5 mg dry wt cellular material/flask and are not corrected for endogenous respiration. Both preparations were starved for 5 days.

M. quinckeanum, #8, and to a much lesser extent with strain #13 of this species. Similar morphological alterations did not occur with the other organisms used. As illustrated in Figure 2, pellets composed of typical mycelial structures obtained from submerged growth cultivation fragmented into much smaller elements. This fragmented material chiefly consisted of microconidia, arthrospores, chlamydospores and a small amount of short hyphal sections. This material resembles the structures reported to appear during the latter stages of growth when dermatophytes are cultivated with continuous shaking (16). When this material is grown in Sabouraud's glucose broth for 2 days on the shaker, intact typical pellets are obtained which possess characteristic mycelial strands as shown in Figure 3.

Use of fragmented cellular elements permitted more accurate transfer of material for metabolic studies. As reported in Table II, it was observed that this disperse form of growth possessed more activity per unit of dry weight than material from the same organism used in the non-fragmented or mycelial state. It was also found that the fragmented mycelial preparation utilized glycine as readily as alanine with respect to oxygen uptake and ammonia production.

TABLE III

Ammonia formation, oxygen uptake, pyruvic acid and L-alanine values by fragmented cellular preparations of Microsporum quinckeanum, #8

Material Tested	Reaction Time (Hours)						
	0	½	1	2	3	4	5
Substrate Flasks							
μl O_2 uptake/flask	0	96	186	337	467	588	695
μM NH_3/ml	.12	.25	.40	.62	.70	.85	.95
μM L-alanine/ml	1.0	.87	.72	.54	.43	.40	.31
μl O_2 uptake/flask	0	66	126	241	341	436	536
μM NH_3/ml	1.05	1.09	1.15	1.25	1.29	1.28	1.27
μM pyruvic acid/ml	1.0	.97	.93	.85	.78	.69	.64
Endogenous Flasks							
μl O_2 uptake/flask	0	70	138	263	378	485	583
μM NH_3/ml	0	—	—	.24	—	—	.30
μM amino acid/ml	0	—	—	0	—	—	0
μM keto acid/ml	0	—	—	0	—	—	0

1.5 ml cells per flask (5mg dry wt). Values not corrected for endogenous.

TABLE IV

Ammonia formation, oxygen uptake, alpha-keto glutaric acid and L-glutamic acid values by fragmented cellular preparations of Microsporum quinckeanum, #8

Material Tested	Reaction Time (Hours)						
	0	½	1	2	3	4	5
Substrate Flasks							
μl O_2 uptake/flask	0	43	90	166	235	292	343
μM NH_3/ml	.05	.12	.19	.31	.50	.59	.70
μM L-glutamic acid/ml	1.0	.84	.68	.57	.50	.45	.40
μl O_2 uptake/flask	0	30	57	103	146	186	224
μM NH_3/ml	1.0	1.09	1.10	1.14	1.17	1.18	1.20
μM alpha-keto glutaric acid/ml	1.0	.95	.88	.88	.87	.87	.85
Endogenous Flasks							
μl O_2 uptake/flask	0	31	63	119	169	218	265
μM NH_3/ml	0	—	—	.17	—	—	.31
μM amino acid/ml	0	—	—	0	—	—	0
μM keto acid/ml	0	—	—	0	—	—	0

1.5 ml cells per flask (5 mg dry wt). Values not corrected for endogenous.

Further investigations were made using fragmented material to determine relationships between ammonia production, oxygen uptake values and residual amino acid from flasks containing 1 μM/ml L-alanine. Equivalent respiratory studies were carried out for pyruvic acid and ammonium sulfate. Oxygen uptake values, residual keto acid and ammonia determinations also were

TABLE V

Ammonia formation, oxygen uptake, oxalacetic acid and L-aspartic acid values by fragmented cellular preparations of Microsporum quinckeanum, #8

Material Tested	Reaction Time (Hours)						
	0	½	1	2	3	4	5
Substrate Flasks							
μl O_2 uptake/flask	0	46	91	175	235	290	339
μM NH_3/ml	.04	.22	.35	.47	.67	.87	1.01
μM L-aspartic acid/ml	1.0	.87	.69	.42	.36	.30	.24
μl O_2 uptake/flask	0	36	77	147	208	262	309
μM NH_3/ml	1.09	1.11	1.26	1.33	1.51	1.60	1.65
μM oxalacetic acid/ml	1.0	.97	.97	.97	.96	.96	.94
Endogenous Flasks							
μl O_2 uptake/flask	0	38	73	141	204	260	310
μM NH_3/ml	0	—	—	.28	—	—	.70
μM amino acid/ml	0	—	—	0	—	—	0
μM keto acid/ml	0	—	—	0	—	—	0

1.5 ml cells per flask (5 mg dry wt). Values not corrected for endogenous.

made on these flasks. Results are reported in Table III.

Similar experiments with L-glutamic and L-aspartic acids were made with corresponding flasks containing ammonium sulfate and alpha-keto glutaric or oxalacetic acid. Results are shown in Tables IV and V.

Enzyme Preparations

Active enzyme systems were obtained using both methods of cell breakage. Pyruvic and glutamic acid dehydrogenases and catalase were noted by the Thunberg reductase technic. L-amino acid oxidase activity could not be demonstrated in non-dialyzed enzyme preparations obtained from cells in various stages of growth or starvation.

Exposure of cells to an amino acid before cell breakage was of no aid. Addition of flavin adenine mononucleotide (.75 μg), flavin adenine dinucleotide (.75 μg), diphosphopyridine nucleotide (100 μg), triphosphopyridine nucleotide (100 μg), mercaptoethanol (0.1 M), and methylene blue (0.000267 M) to Warburg vessels did not promote L-amino acid oxidase activity. An alteration in pH of reaction mixtures from 7.0 to 8.5 in phosphate buffer (0.066 M) with added cofactors produced no change in results.

As we were unable to obtain an active L-amino acid oxidase system for stoichiometric studies, sodium selenite (.005 M/Warburg vessel) was added to substrate reaction flasks to determine the product of L-alanine deamination. A derivative corresponding to the pyruvic acid hydrazone was observed in chromatograms of 2,4-dinitrophenylhydrazine derivatives of the reaction mixture.

DISCUSSION

Certain observations by Bentley (1), and Burack and Knight (2), concerning L-amino acid oxidase systems in dermatophytes have been confirmed and extended by this investigation. Six of the seven organisms used readily produced ammonia and took up oxygen in the presence of separate amino acid substrates. L-alanine was oxidized more readily than any other amino acid tested and ammonia production in the presence of L-aspartic acid was high. The strain of *E. floccosum* tested utilized amino acids only slightly, even when concentrated suspensions were used. This latter observation does not agree with the findings of Burack and Knight (2) with respect to ammonia production. We have found great differences in amino acid utilization by the dermatophytes tested and our results emphasize the

diverse metabolic rates and abilities possessed by these organisms. From these varied results, it appears that ability to produce ammonia is not a factor of primary significance in degree of virulence, or in the pathogenic process as suggested by others (2).

We have shown that a progressive decrease in amount of amino acid present occurs concurrently with an increase in ammonia production and microliters of oxygen taken up by the dermatophytes tested. In each case, more oxidation of the amino acid occurred than was required for deamination alone.

During this investigation it was found that starvation of mycelial pellets in phosphate buffer plus added L-alanine provided a preparation which readily utilized amino acids. This result was especially true in the case of *M. quinckeanum*, #8. The increase in utilization of substrates may not be due to an increase in L-amino acid oxidase activity. This oxidase system appears to be a constitutive enzyme even though amino acid utilization can be increased by exposure of cells to L-alanine during starvation. It is suggested that increase in amino acid utilization may be due to the reduction of endogenous reserves to a critical level. Thus, the addition of exogenous amino acid in the form of L-alanine during starvation provides the organisms with a source of carbon, nitrogen and energy to replenish cellular reserves. Addition of penicillin and streptomycin in the concentrations used, according to observations of others (8), neither affects dermatophyte growth adversely nor do they suppress deaminase activities according to our results.

Unlike the observations of Bentley (1), we were unable to obtain active cell-free extracts for amino acid oxidase studies using acetone preparations.

Various approaches have been used to obtain uniform mycelial preparations for quantitative metabolic studies of dermatophytes. Separation of pellets of uniform size (17) is tedious, and homogenization in a blender (18, 19, 20, 21) may reduce their physiological activity markedly. One of the eight organisms used in this study, *M. quinckeanum*, #8, provided a fragmented preparation during an extension of starvation time. This dispersal of mycelia from pellet formation may be due to initial stages of autolysis and thus induce fragmentation. However, these cellular elements possessed more physiological activity than did intact pellets. Assimilation of exogenous amino acid at this stage during starvation may take place more readily, and could be partially responsible for the increase in activity.

The uptake of oxygen below endogenous levels by fragmented mycelia of *M. quinckeanum*, #8, in the presence of ammonia and pyruvic acid or alpha-keto glutaric acid was an unexpected observation. The presence of these keto acids may cause a concomitant decrease in the rate of utilization of endogenous materials. L-alanine disappearance coincides with ammonia production. L-aspartic and L-glutamic acids, however, may be assimilated by the fragmented preparation. Since a 1:1 ratio of keto acid and ammonia utilization was not obtained, assimilation of these materials into protein does not appear to occur.

SUMMARY

L-amino acid oxidase activity can be demonstrated in certain superficial dermatophytes after starvation while shaking in the presence of buffer. Endogenous-amino acid substrate separation in respiratory studies may be obtained, also, by the addition of L-alanine during shorter periods of starvation.

A wide range of amino acids are utilized by the dermatophytes studied. Most of these organisms produced ammonia from a number of amino acids but with differing avidity. The ability to oxidize and to deaminate substrate differed according to the amino acid and with the organism.

A strain of dermatophyte was found to undergo fragmentation without loss in physiological activity thereby permitting accurate replication in quantitative metabolic studies.

A definite correlation concerning utilization of ammonia and other products of deamination from various amino acids could not be obtained.

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